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TOPIC 09 – Angiogenesis

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0299

N, N-diethyl-m-toluamide (DEET) stimulate cellular processes leading to angiogenesis *via* the activation of muscarinic receptors

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Insect repellents containing DEET are highly effective through an inhibition of acetylcholinesterase (AChE) activity and these pesticides have been reported to play a role in tumor development. Different mechanisms can lead to stimulate angiogenesis, such as the inhibition of AChE activity. The present study was designed to test the potential effect of DEET on different processes leading to angiogenesis on human endothelial cells (HUVECs) including proliferation, adhesion and migration through an involvement of cholinergic receptors. DEET was incubated for 24h on HUVECs at concentrations similar to that found in plasma of exposed individuals (10-5M) or in the environment (10-8M). VEGF(20 ng/mL) was used as a positive control. Proliferation was analyzed by using CyQUANT Cell Proliferation Assay Kit. Evaluation of adhesion was performed using crystal violet staining. Cell migration was evaluated using Transwell migration kit. Analysis of both superoxide anion (O_2-) and nitric oxide (NO) productions were performed using electronic paramagnetic resonance technique. Quantitation of AchE activity was performed by Ellman colorimetric assay. The two concentrations of DEET increased cell proliferation. Moreover, it potentiated cell migration that was associated with enhanced MMP2 activity and endothelial cell adhesion through an enhancement of FAK phosphorylation and stress fibers. Although it did not modify O_2- production, DEET increased NO production through an increase of peNOS-Ser/peNOS-Thr ratio. All of these cellular processes were partially prevented by methoctramide and pFHHSiD, M2 and M3 antagonists, respectively and DEET inhibited endothelial AChE activity. Altogether, these data highlight that DEET modulates cellular angiogenic processes through an activation of muscarinic receptors. In addition to their toxic effects on nervous system, this study underscores that DEET may affect the generation of vascular network that could potentiate proliferative diseases.

0340

Targeting VEGFR1 on endothelial progenitors modulates their differentiation potential

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Objectives: We studied whether plasma levels of angiogenic factors and endothelial markers in coronary artery disease patients or undergoing cardiac surgery are modified, and whether those factors modulate endothelial progenitor's angiogenic potential.

Methods and Results: 143 patients plasmas from two different studies were analyzed (30 coronary artery disease patients, 30 patients with stable angina, coupled with 30 age and sex-matched controls; 53 patients underwent cardiac surgery). Among factors screened, only placental growth factor (PlGF) was found significantly increased in these pathological populations. PlGF-1 and -2 were then tested on human endothelial colony forming cells (ECFCs). We found that PlGF-1 and -2 induce VEGFR1 phosphorylation and potentiate ECFCs tubologenesis in vitro. ECFCs VEGFR1 was further inhibited using a specific small interfering RNA (siRNA) and the chemical compound 4321. We then observed that the VEGFR1-siRNA and the 4321 compound decrease ECFCs tubologenesis potential in vitro. Finally we tested the 4321 compound in the preclinical Matrigel®-plug model with C57Bl/6J mice, and found that 4321 inhibited the plug vascularization, attested by the haemoglobin content and the VE-Cadherin expression level.

Conclusion: PlGF plasma levels were found increased in cardiovascular patients. Disrupting PlGF/VEGFR1 pathway could modulate ECFCs induced tubulogenesis, the cell type responsible for newly formed vessels in vivo.

0154

Frizzled 7 is required for post natal angiogenesis

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Vessel formation requires a precise orchestration of a serie of morphometric and molecular events controlled by a multitude of angiogenic factors. Growing evidence has shown that Wnt/Fzd proteins are expressed in vascular cells and are directly involved in blood vessel development by regulating endothelial cells proliferation, polarity and vascular branching morphogenesis. In this study, we analyzed the consequences of *fzd7* receptor deletion on postnatal vessel formation in mice retina by developing a Cre-Lox strategy. Tie2Cre+/*fzd7*$^{f/Wt}$ mice were crossed with *fzd7*$^{f/f}$ mice to generate Tie2Cre+/*fzd7*$^{f/f}$ (*fzd7*^{ECKO}) mice where *fzd7* gene is deleted specifically in endothelial cells. These mice are viable and have been used and compared with WT mice.

The retinas of pups sacrificed 7 days after birth were immunostained (CD31, NG2 and SMA) to study the kinetics of vascular plexus formation. We observed that *fzd7*^{ECKO} mice had a delay in vascular network formation compared to WT (-18% of vascular radial extension, $p<0.01$). In the *fzd7*^{ECKO} retinas, vascular density and the branching of this network were significantly increased. Confocal microscopy analysis of tip-cell at the migration front showed a strong impairment of tip-cell phenotype in the *fzd7*^{ECKO} retina vs. WT. We observed a significant increase in tip cell number (+40%, $p<0.05$) and filopodia number per tip cell (+48.5%, $p<0.05$). These filopodia were disorganized and misguided.

Due to the impairment of tip cell phenotype in *fzd7*^{ECKO} mice, we analyzed *in vivo* and *in vitro*, the expression of Notch partners by quantitative RT-PCR. *In vivo*, *fzd7* deletion induced a decrease in Notch ligands (Dll4, Jag1), Notch receptors (Notch1, Notch4) and Hey2 expression. Moreover, *in vitro* siRNA strategy against *fzd7* on murine EC confirmed these results. This study shows that *fzd7* is required for the formation of vascular network and that *fzd7* appears to regulate the phenotype of the tip cells.

0213

ANGPTL4-αVβ3 integrin interaction counteracts VEGFR2 signaling and hypoxia-induced vascular leakage

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We previously showed that ANGPTL4-mediated vasculoprotection is crucial for secondary cardioprotection during AMI and might constitute a relevant target for therapeutic intervention as it contributes to decrease hemorrhage, edema, inflammation « no-reflow » phenomenom and infarct size in ischemia-reperfusion models. The aim of this study is to decipher the mechanisms through which ANGPTL4 preserves vascular integrity during ischemic diseases.

We show that vascular leakage is increased in angptl4-deficient mice during choroidal neovascularization. Recombinant human ANGPTL4 inhibits hypoxia–induced vascular permeability and increases adherens and tight junctions integrity. Using the Surface Plasmon Resonance (SPR) binding technique and a vascular permeability assay combined with a blocking antibody strategy, we show that this inhibition is mediated through ANGPTL4 binding to integrin αVβ3. Finally, biochemical analyses reveal that ANGPTL4 modulates Src recruitment and activation at integrin αVβ3, VEGFR2 and VE-Cadherin complexes and decreases VEGF signaling.

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ANGPTL4 acts through binding to αVβ3 integrin in order to counteract vascular permeability. Mechanistically, ANGPTL4-αVβ3 integrin interaction increases Src recruitment to αVβ3 complexes, thereby leading to a diminished Src signaling at VEFGR2. This translates to decreased breakdown of the VEGFR2/VE-cadherin complex and an increased stability of tight and adherens junctions in endothelial cells.

0046

Gli3 promotes ischemia-induced angiogenesis at least in part by regulating post-natal myogenesis

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Background: Skeletal muscle regeneration occurs after many traumas and diseases including congenital myopathies, ischemic diseases and sports related injuries. Understanding mechanism underlying skeletal muscle repair is then of great interest. The embryonic Hedgehog (Hh) signaling was shown to be still active in adults and to regulate myogenesis and angiogenesis, two crucial processes for muscle regeneration. This study further characterizes cellular and molecular mechanisms coordinating angiogenesis and myogenesis and the role of the Hh transcription factor Gli3.

Results: Because we recently found that Gli3 is essential for ischemic muscle repair, we used mice in which Gli3 has been specifically disrupted in ECs (Tie2-Cre; $Gli3^{Flox/Flox}$) or in skeletal myocytes (HSA-Cre^{ERT2}; $Gli3^{Flox/Flox}$) and found that Gli3 KO in ECs does not significantly impairs hind limb ischemia-induced muscle repair while Gli3 KO in myocytes severely delays ischemia-induced myogenesis. Moreover, this study shows that angiogenesis is also significantly impaired in HSA-Cre^{ERT2}; $Gli3^{Flox/Flox}$ mice demonstrating that impaired myogenesis, indirectly affects ischemia-induced angiogenesis. The role of Gli3 in myocytes was then further investigated *in vitro*. We found that Gli3 promotes myoblast differentiation through Myf5 regulation; moreover Gli3 expression level regulates several proangiogenic factors including Angiopoietin1 and Thymidine phosphorylase in myoblasts which indirectly regulate EC proliferation. Finally this study shows for the first time that Hh signaling and more particularly Gli3 mRNA expression can be regulated by myogenic growth factors including IGF-1.

Conclusion: The study shows that ischemia-induced angiogenesis is tightly regulated by myogenesis at least in part through Gli3 regulation in myocytes and suggest that myogenic therapy can be considered to promote ischemia-induced angiogenesis and muscle repair.

0195

Palmitic acid promotes pro-oxidant adaptor protein p66Shc expression and affects vascularization factors in pro-angiogenic cells

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Type-2 diabetes is associated with impaired neovascularisation, which involves dysfunction of circulating pro-angiogenic mononuclear cells (CAC). Elevated plasma free fatty acid (FFA) levels such as palmitic acid (PA), and oxidative stress may directly affect CAC numbers and function. Interestingly, a recent study pointed out the lifespan regulator p66Shc, a pro-oxidant adaptor protein as a modulator of KLF2 (Krüppel-like factor 2), a beneficial actor of CAC and endothelial function. We thus aimed to follow the molecular impact of high levels of FFA in CACs in vitro.

CACs were differentiated from peripheral blood mononuclear cells in an endothelial growth medium. At day 6, adherent cells pretreated or not with 30μM resveratrol (which promotes endothelial KLF2) for 3 days, were submitted to a 24h treatment with PA (200 or 400 μM) or vehicle. Q-PCR analysis was performed. Cytoplasmic reactive oxygen species production was measured with the oxidative sensitive fluorescent probe CM-DCF. Protein levels of Tie2 and VEGFR2 were measured by FACS and Imagestream cytometer.

We observed that while KLF2 expression tended to decrease, PA markedly and dose-dependently enhanced p66Shc mRNA expression (6 fold). PA did not markedly affect CAC number but a rise in oxidative stress was evidenced by higher number of CM-DCF positive cells. Resveratrol pretreatment reversed PA-induced oxidative stress and tended to normalize gene expression levels. Interestingly, PA-induced p66shc increase correlated with a large rise in mRNA expression of VEGF-A (10 fold). Conversely, surface membrane expression of VEGFR2 and Tie2 decreased after PA treatment.

We show for the first time that high levels of FFA can directly impair CAC function, possibly *via* an increase in p66Shc-induced oxidative stress leading to a deregulated VEGF-A over-expression. The associated decrease in major receptors involved in neovascularization, such as VEGFR2 and Tie2, highlights the potent deleterious action of PA in this process.

0188

Role of lysyl oxidase like-2 in tumor angiogenesis

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Hypoxia-driven remodeling of the microenvironment, consisting in extracellular matrix (ECM) and various cell types is a regulated process involved in angiogenesis and tumor progression. Lysyl oxidase like-2 (LOXL2) is upregulated in many cancers in both tumor and stromal cells and involved in ECM crosslinking. We demonstrated that : 1/LOXL2 secretion is upregulated by hypoxia in endothelial cells and accumulates in the ECM ; 2/LOXL2 regulates sprouting angiogenesis and type IV collagen assembly in the endothelial basement membrane during developmental vascularisation (Bignon, Blood 2011). Inhibition of extracellular LOXL2 impedes the development of pathologic microenvironment and thereby controls cancer progression (Barry-Hamilton, Nat. Med. 2010). We therefore aimed to investigate the role of LOXL2 in tumor angiogenesis.

As metastatic progression is dependent on the tumor microenvironment, we carried out the analysis of *loxl2* mRNA in a collection of human metastatic tissues. Our data evidenced a strong expression of *loxl2* mRNA in adrenal metastasis from renal clear cell carcinoma.

In parallel, we assessed the functional involvement of LOXL2 in regulating angiogenesis using:

1/ An *in vitro* 3D angiogenesis model of HUVEC spheroids included in a mixed Matrigel/fibrin gel. We show that the length of endothelial tubes is increased in comparison to control conditions when recombinant LOXL2 is added into the gel.

2/An *in vivo* angiogenesis model in mice, based on subcutaneous injections of Matrigel/fibrin that contains or not LOXL2. Our earliest results show an increased vascularisation of the LOXL2-containing Matrigel plugs compared to control, as evidenced by quantifying the hemoglobin content at Day 7.

Altogether, these results support that LOXL2 secreted in tumor microenvironment is involved in regulating angiogenesis. Further studies are ongoing to determine the synergy between LOXL2-induced angiogenesis, remodeling of the microenvironment and tumor progression.

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